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TITLE: Apoptosis Induction by Targeting Interferon Gamma Receptor 2 (IFNgammaR2) in Prostate Cancer:  
Ligand (IFNgamma)-Independent Novel Function of IFNgammaR2 as a Bax Inhibitor

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  In our preliminary study, we found that IFN $\gamma$ R2 has previously unknown function as an inhibitor of Bax. Bax is a key mediator of apoptosis. We found that IFN $\gamma$ R2 is overexpressed in prostate cancer, and we hypothesize that abnormally high level of IFN $\gamma$ R2 confers apoptosis resistance of prostate cancer. In this project, we will investigate the role of IFN $\gamma$ R2 in drug resistance of prostate cancer and explore the development of strategies that can activate Bax-induced apoptosis in prostate cancer by inactivating IFN $\gamma$ R2. In Year 3, we planned to determine what kind of cell type(s) in prostate cancer tissue expresses IFN $\gamma$ R2 by performing immunohistochemistry. Another important proposed experiment is to determine whether IFN $\gamma$ R2 expression profile (expression levels and expression type (cytosol or membrane expression, or cell type specific staining) can be used as a biomarker to predict the clinical outcome. In this report, we show that IFN $\gamma$ R2 is expressed in a particular group of basal cells in prostate of patients who had recurrence. IFN $\gamma$ R2 positive cells were not detected in luminal cells or luminal cell type cancer cells. Using prostate cancer cell lines, we found that IFN $\gamma$ R2 expression increases according to the progression of malignancy, i.e. from androgen-dependent state to androgen-independent and metastatic state. These results suggest that elevation of IFN $\gamma$ R2 expression is correlated with progression of prostate cancer.					
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## Introduction

In our preliminary study, we identified interferon  $\gamma$  receptor 2 (IFN $\gamma$ R2) as a Bax suppressor using yeast-based functional screening of Bax inhibiting proteins. Bax is a key mediator of apoptosis which is essential for chemotherapy- and radiation-induced apoptosis of prostate cancer cells. We found that IFN $\gamma$ R2 levels are abnormally elevated in prostate cancer cell lines. Short hairpin (sh) RNA-mediated knockdown of IFN $\gamma$ R2 was able to increase chemotherapy-induced apoptosis rate significantly in prostate cancer cells, suggesting that IFN $\gamma$ R2 is a chemo-resistant factor in prostate cancer cells. Although IFN $\gamma$ R2 was previously known as a receptor of IFN $\gamma$  which is an anti-tumorigenic cytokine, our preliminary data suggest that IFN $\gamma$ R2 expresses its anti-apoptosis (anti-Bax) activity independent from IFN $\gamma$  and IFN $\gamma$  signaling. Importantly, we found that IFN $\gamma$ R2 is expressed in mitochondrial membranes and endoplasmic reticulum (ER) membranes, but not on the plasma membranes of prostate cancer cells. Since we found that IFN $\gamma$ R2 can directly interact with Bax in vitro, we hypothesize that IFN $\gamma$ R2 confer apoptosis resistance of prostate cancer by directly binding and inhibiting Bax in intracellular membrane such as endoplasmic reticulum (ER) and mitochondria (Fig.1).

In this 3 years DOD Prostate Cancer Research IDEA project, the following Tasks will be examined to develop novel anti-prostate cancer therapy as well as to establish IFN $\gamma$ R2 as a diagnostic marker to predict the chemo-resistance of prostate cancer.

**Task 1:** To determine the mechanism of Bax inhibition by IFN $\gamma$ R2, and to develop anti-IFN $\gamma$ R2 peptide that enhances Bax activation. (Months 1-24)

**Task 2:** To identify the subtype of prostate cancer that can be effectively treated by IFN $\gamma$ R2-targeting technologies (Months 13-36)

**Task 3:** Determination of the mechanism of abnormal expression of IFN $\gamma$ R2 in prostate cancer (Months 13-36)

In the first year, Task 1 was the main part of our study and we were able to obtain information about the binding domains of IFN $\gamma$ R2 and Bax, as reported in the last progress report. In Year 2, experiments of Task 2 and Task 3 have started, and we obtain important results that will help us to develop new anti-prostate cancer strategy based on novel anti-apoptotic activity of IFN $\gamma$ R2. Especially, we showed that NF $\kappa$ B inhibitor (Parthenolide) was able to decrease IFN $\gamma$ R2 in prostate cancer cell lines, supporting our hypothesis that NF $\kappa$ B is one of the factors increasing expression of IFN $\gamma$ R2 in prostate cancer. In Year 3, we were able to start experiments in Task 3 since we obtained the first set of patient specimen (paraffin block) to investigate the subtype of prostate cancer expressing IFN $\gamma$ R2. In this Year 3 report, we show results suggesting that IFN $\gamma$ R2 expressing prostate cancer cells arise from basal cell, rather than luminal cell in prostate tissue. Our results imply that tumor initiating cells (or cancer stem cells) that are known to emerge from basal cells may express high level of IFN $\gamma$ R2 to survive from apoptotic stresses.

## Body (Methods, Results and Discussion)

### Results and Discussion

**Task 2: IRB protocol was approved, patient's samples were located, and first experiment were performed (Figs. 2-4)**

In Task 2, we proposed to determine the correlation between IFN $\gamma$ R2 expression and clinical outcome by examining IFN $\gamma$ R2 expression of prostate tissue specimen obtained from patients with

clinical record. After several months of attempts to obtain approval from local IRB committee, we finally obtained approval in the end of 2014. We identified 100 patients' paraffin blocks with solid clinical record who received prostatectomy and radiation therapy afterward. From January 2015, we requested our Pathology Department to locate and provide paraffin blocks for our research project, and we obtained the first set of 7 patients samples (3 were cured, 4 had recurrence). We performed immunohistochemistry of IFN $\gamma$ R2 using these 7 paraffin blocks and obtained interesting results as described in the next section. Unfortunately, it takes very long time for Pathology Department to locate and prepare slide sections, and we are still waiting the next set. We hope to complete the analysis of 100 patient samples by the summer of 2016.

### **Task 2: Significant IFN $\gamma$ R2 expression was detected in prostate cancer cells of patients who experienced recurrence after prostatectomy and radiation therapy.**

In Figs. 2-3, representative results are shown. Fig.2 (A and B) show IFN $\gamma$ R2 expressing cells were clearly detected in prostates of patients with recurrence (in all 4 patients examined), but IFN $\gamma$ R2 expressing cells were NOT always found in prostates of patients who did not have recurrence (2 out of 3 patients did not have IFN $\gamma$ R2 positive cells) (Fig. 2C). Interestingly, IFN $\gamma$ R2 was detected in basal cells, but not in luminal cells. Furthermore, IFN $\gamma$ R2 is not expressed in all basal cells, but only in basal cells that show hyperplasia destructing the tube structure of prostate gland (Fig. 2A) or in basal cells in a few prostate glands that still maintains normal structure (Fig. 2B). We speculate that Fig.2B shows the emergence of tumor-initiating basal cells in particular area, and Fig. 2A shows the example of pre-tumorigenic growth of basal cells. In Fig. 3, other pictures of IFN $\gamma$ R2 positive prostate cells are displayed in the order of the progression of prostate cancer. Fig.3D shows the example of the destruction of luminal structure by abnormal growth of basal cells that express IFN $\gamma$ R2. Fig.3E is the results in metastatic prostate cancer section showing that majority of cancer cells express high level of IFN $\gamma$ R2. Interestingly, IFN $\gamma$ R2 was not detected in luminal cell type prostate cancer (see Fig. 2C) suggesting that IFN $\gamma$ R2 expression may decrease when basal cell differentiates into luminal cells. Since IFN $\gamma$ R2 protect prostate cancer cells from apoptosis, IFN $\gamma$ R2 (-) luminal cell type cancer cells are likely treatable (i.e. relatively easy to induce apoptosis) cancer and IFN $\gamma$ R2 (+) basal cells may represent apoptosis-resistant cancer initiating cells or cancer stem cells (Fig. 4).

### **Task 3: IFN $\gamma$ R2 expression increases according to the progression of malignancy of prostate cancer cells (Figs. 5-6).**

Please see Fig.5. We examined expression levels of IFN $\gamma$ R2 in widely used prostate cancer cell lines. These cell lines were originated from the same cell line to investigate the molecular mechanism of progression of prostate cancer, i.e. from androgen-dependent state (LNCaP) to androgen-independent state (C4-2), and to bone metastatic state (C4-2B). Interestingly, IFN $\gamma$ R2 levels increased according to the progression of malignancy in these prostate cancer cells. It has been known that C4-2B has constitutive active androgen receptor (AR). Therefore, elevated activity of AR may contribute to the increased expression of IFN $\gamma$ R2.

Since C4-2B expresses high level of IFN $\gamma$ R2, we speculated that IFN $\gamma$ R2's role to suppress cell death is significant in this cell line. In fact, IFN $\gamma$ R2 knock down significantly slowed down the growth of C4-2B and induce spontaneous cell death as seen in Fig. 6C. These results suggest that IFN $\gamma$ R2 has a significant role to suppress apoptosis in aggressive prostate cancer that shows androgen independency and metastatic activity.

## **Methods:**

### *shRNA-mediated down regulation of IFN $\gamma$ R2 in C4-2B cell line*

IFN $\gamma$ R2 targeting shRNA was introduced into cells by using lentivirus transfection system (Thermo Fisher Scientific, USA). Control shRNA encodes shRNA against Green Fluorescent Protein (GFP) that does not exist in human cells. Cells successfully transfected by lentivirus were selected by puromycin, and cell lysates were collected to determine IFN $\gamma$ R2 protein expression.

### *Immunohistochemistry of human prostate cancer tissue microarray.*

Human prostate cancer tissue microarray was purchased from BioMax (Maryland, USA). Immunohistochemistry of IFN $\gamma$ R2 was performed by the standard methods explained in detail in Abcam website (<http://www.abcam.com/index.html?pageconfig=resource&rid=13046>). Antibodies used in these experiments is: IFN $\gamma$ R2 (Abcam, #ab77246).

### *Cell culture and cell lysate preparation for Western blot*

LNCaP, C4-2, and C4-2B cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and 1% penicillin/streptomycin. Cell lysates were prepared by solubilizing cells using 1% NP40 containing HEPES buffer. Insoluble fraction was removed by centrifuge separation (14k rpm for 20n min at 4C). For the analysis of protein expression, cell lysates containing 10 ug protein were used. SDS-PAGE was performed by using 4-20% gradient gel, and immuno-detection was performed by ECA Chemical luminescence detection kit (Amersham).

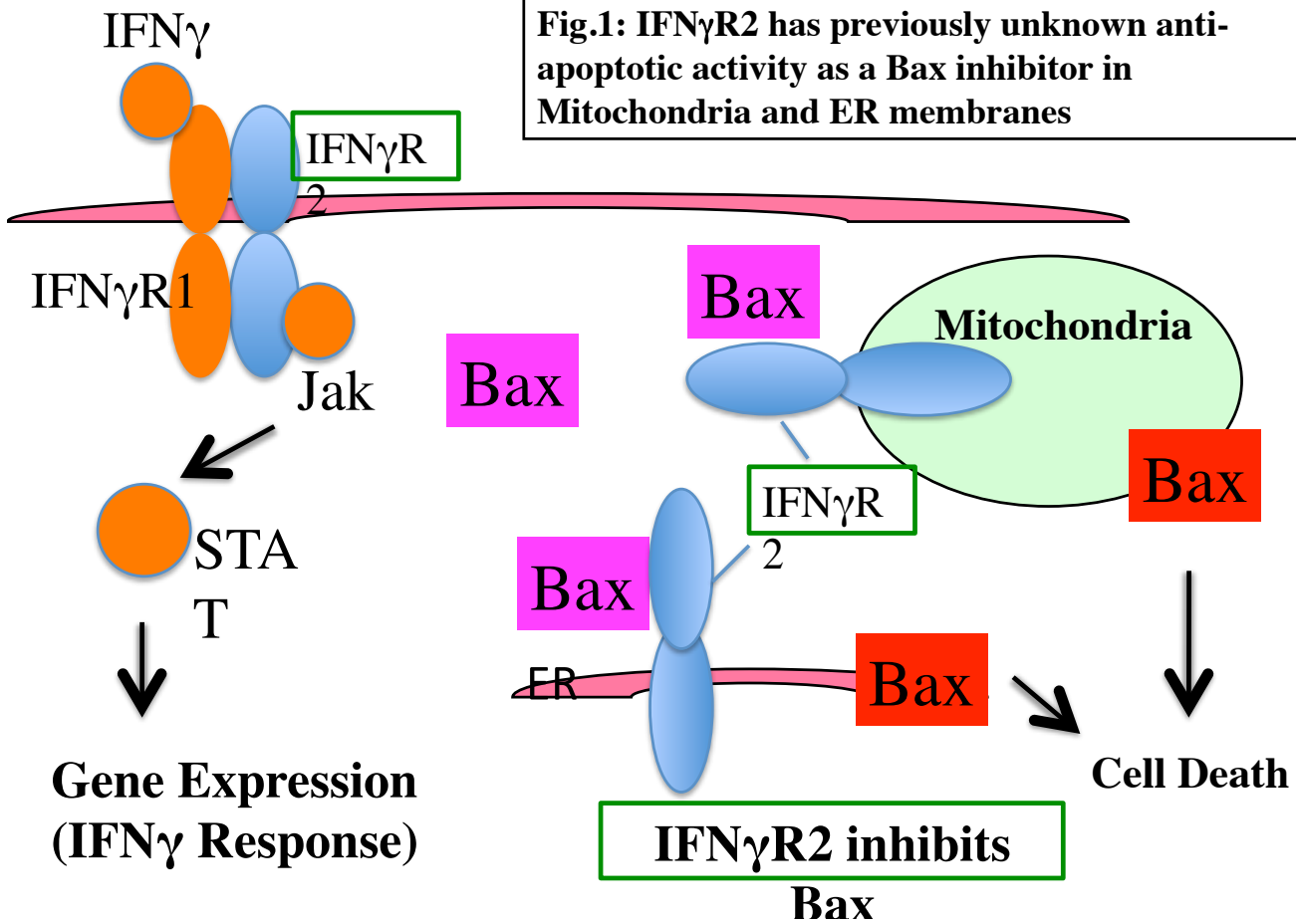
## **Key Research Accomplishment**

1. Existence of subtypes of prostate cancer expressing high levels if IFN $\gamma$ R2 was confirmed by using human prostate cancer tissue micro array and publically available gene expression data base.
2. We found that IFN $\gamma$ R2 expressing cells are basal cells, but not luminal cells.
3. We found that IFN $\gamma$ R2 expression level increase according to the progression of malignancy of prostate cancer.

## **Reportable Outcome**

We are preparing an article to be submitted at the end of 2015 or early in 2016 that will report the effects of shRNA in C4-2B prostate cancer cells as well as striking IFN $\gamma$ R2 staining pattern in prostate cancer tissue (only basal cell in particular area become IFN $\gamma$ R2 positive). We are also planning to submit another paper reporting the outcome of the immunohistochemical analysis of 100 patient specimen whether IFN $\gamma$ R2 staining pattern can be used as a prediction marker for the recurrence after prostatectomy and radiation therapy.

**Fig.1: IFN $\gamma$ R2 has previously unknown anti-apoptotic activity as a Bax inhibitor in Mitochondria and ER membranes**



## Figure 2

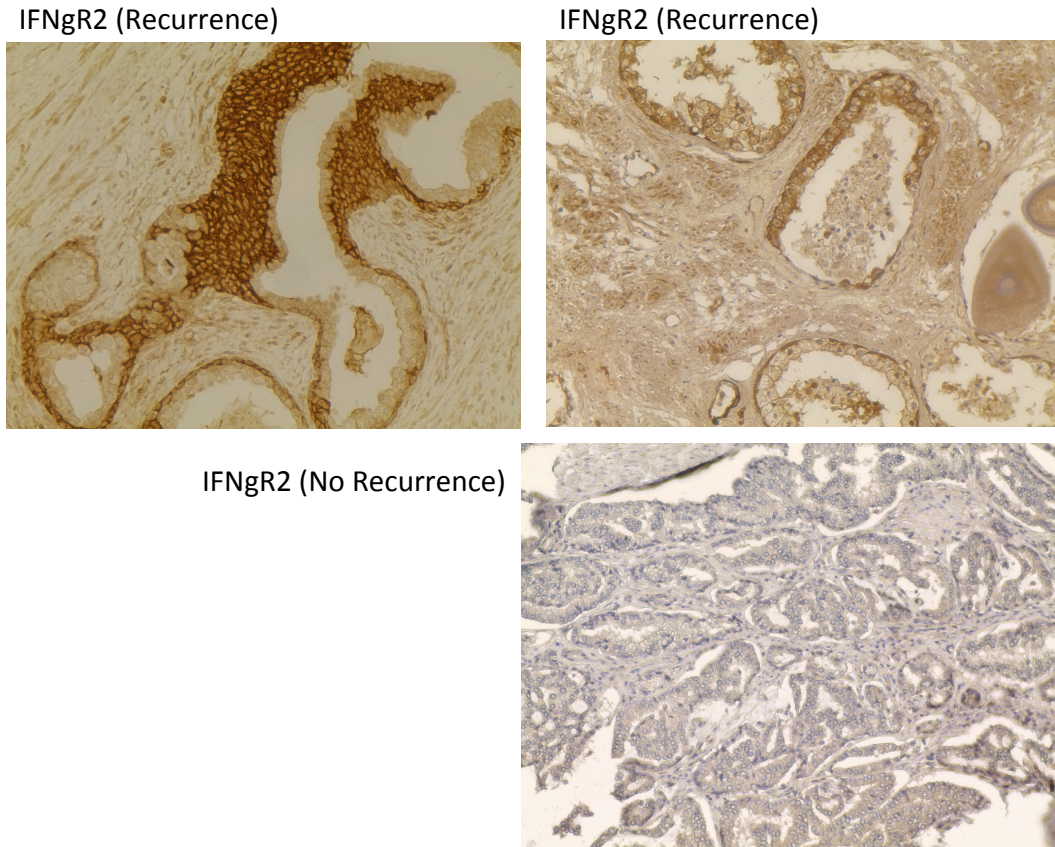


Fig.2. Immunohistochemistry of IFN $\gamma$ R2 in human prostate. A and B: IFN $\gamma$ R2 staining (brown cells, stained by HRP (Horse Radish Peroxidase)-DAB (Diaminobenzidine)) is detected in basal cells, but not in luminal cells or other cell types in prostate. C: IFN $\gamma$ R2 was not detected in prostate cancer area. Note: A and B are results of prostates from patients with recurrence. C is from a patient with no recurrence.

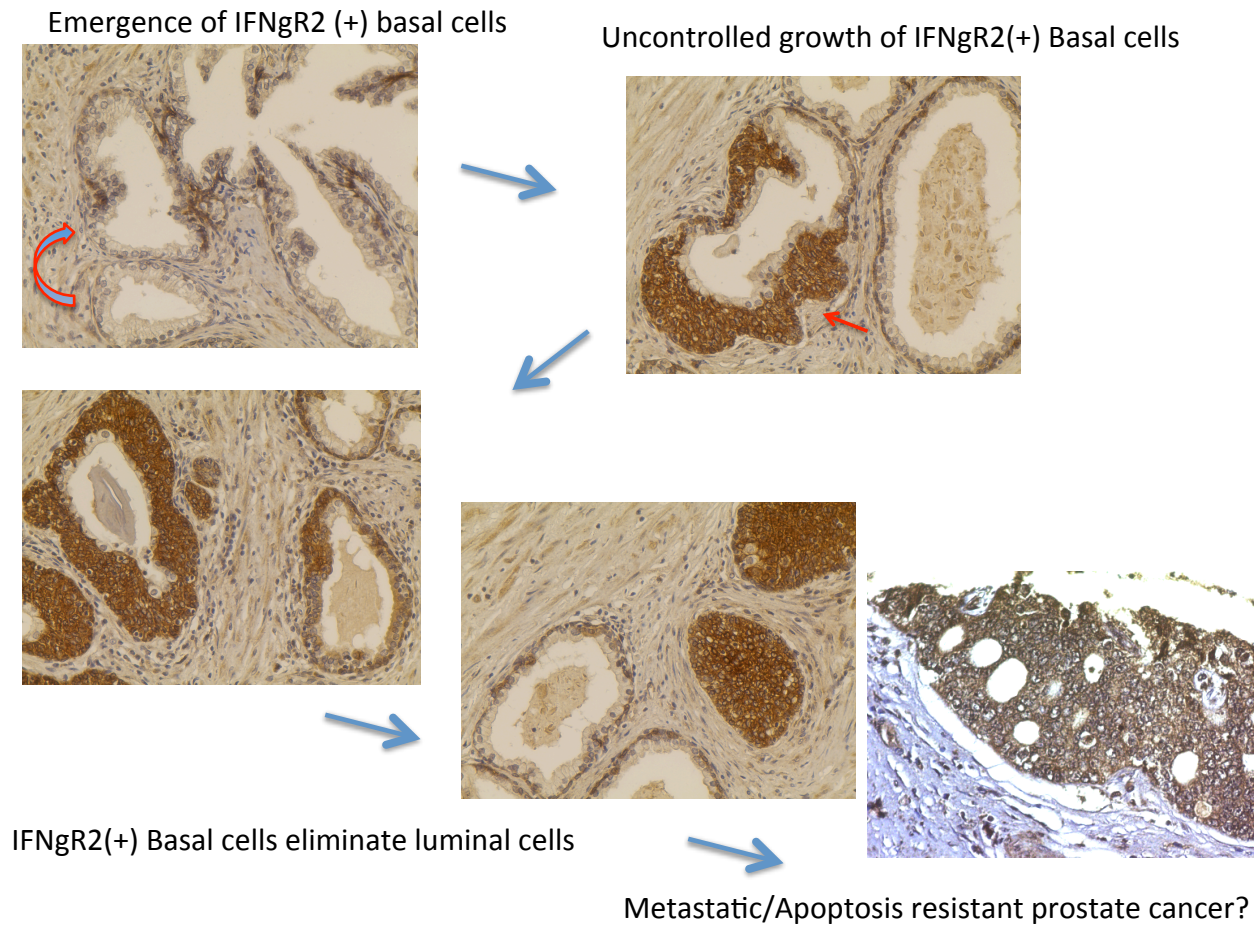
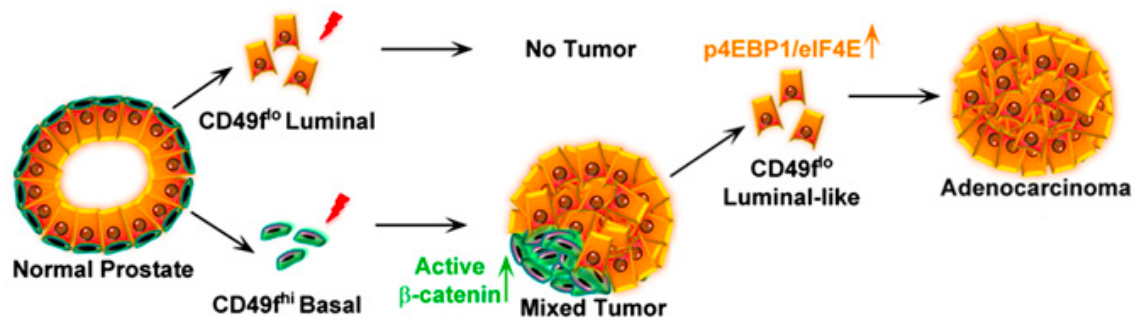


Fig.3. Summary of immunohistochemistry experiments of IFN $\gamma$ R2 in prostates of patients with recurrence. From A to D, an emergence and growth of abnormal basal cells expressing IFN $\gamma$ R2 are shown. E shows IFN $\gamma$ R2 positive prostate cancer cells that showed bone metastasis.

**Fig.4**

***IFNgR2 is expressed in a subgroup of basal cells showing hyperplasia***

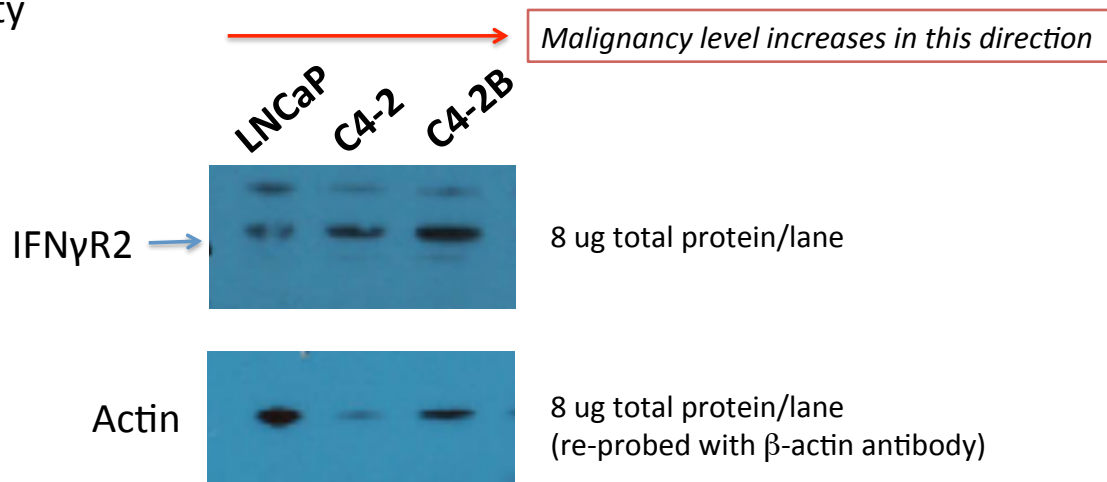


Basal cell acquires IFNgR2 overexpression, and become tumor initiating cells??

Model of human prostate cancer initiation and propagation by distinct phenotypic cell populations (Modified from *Stoyanova et al. PNAS 2013*)



Fig.5: IFN $\gamma$ R2 expression levels become higher when prostate cancer cell line (LNCaP) acquire androgen-independency and bone metastasis capability



\*LNCaP has a Jak1 inactivating mutation, and thus IFN $\gamma$  does not activate canonical IFN $\gamma$  signaling pathway.

C4-2 and C4-2B cell lines were generated from LNCaP cell line, which is an androgen-dependent cell line. C4-2 is androgen-independent, and C4-2B is androgen-independent and bone metastatic cell lines. These 3 cell lines are used to study the mechanism of prostate cancer progression.

**Fig.6 IFNgR2 shRNA suppressed C4-2B growth**

